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14. ABSTRACT Paget's disease of bone (PDB) affects 2-3% of the population over 60 years of age. SQSTM1/p62 UBA domain mutation ($p62^{P392L}$) is widely identified in PDB and has been shown to increase osteoclastogenesis. Further, paramyxovirus are implicated in PDB and measles virus nucleocapsid (MVNP) has been shown to induce pagetic phenotype in osteoclasts. However, the molecular mechanisms underlying $p62^{P392L}$ and MVNP stimulation of osteoclast differentiation in PDB are unclear. Earlier, we showed retroviral expression of MVNP in osteoclast (OCL) progenitor cells from osteoclast inhibitory peptide (OIP-1) transgenic mouse showed a significant decrease in OCL formation/bone resorption area. Recently, we established microarray profiling of differential gene expression in p62 wild-type, non-UBA domain mutation in exon-7 (A381V) and UBA domain mutation in exon-8 (P392L) and MVNP transduced human bone marrow derived preosteoclast cells. We thus identified MVNP induced signal regulatory protein beta 1 (SIRPβ1) during osteoclast differentiation. Also, we demonstrated that mutant $p62^{P392L}$ expression results in accumulation of polyubiquitinated TNF receptor-associated factor, TRAF6 and elevated levels of phospho-IκB during osteoclast differentiation. Therefore, OIP-1 inhibition of $p62^{P392L}$ mutant and MVNP regulated gene expression during osteoclast differentiation provides new insights into the therapeutic targets to control elevated osteoclast activity in PDB.

15. SUBJECT TERMS

Paget's disease (PDB), measles virus nucleocapsid (MVNP) , p62, osteoclast, RANK ligand (RANKL)

Table of Contents

Introduction

Paget's disease (PDB) affects approximately 2-3 million people over the age 60 years in the United States. Both viral and genetic factors have been implicated in the pathogenesis of Paget's disease. Mutations (P392L) in the ubiquitin-associated (UBA) domain of sequestosome 1 (SQSTM1/p62) gene have been widely identified in these patients (1). Osteoclast is the bone resorbing cell. Paget's disease is characterized by markedly increased osteoclast formation and excess bone resorption. These abnormal osteoclasts contain paramyxo-viral nuclear inclusions and antigens. We previously detected expression of measles virus nucleocapsid (MVNP) transcripts in osteoclasts from patients with Paget's disease. Also, we have shown that MVNP gene expression in normal human osteoclasts precursors results in pagetic phenotype in osteoclasts (2). RANK ligand (RANKL), a critical osteoclast differentiation factor expressed by marrow stromal/preosteoblast cells is increased in PD (3). We have previously characterized the Ly-6 family member, osteoclast inhibitory peptide-1 (OIP-1/hSca) inhibition of osteoclast formation and bone resorption activity. Furthermore, targeted over-expression of OIP-1 in the osteoclast lineage develops an osteopetrosis bone phenotype in mice due to inhibition of osteoclast formation/bone resorption activity in vivo (4). Also, retroviral expression of MVNP in osteoclast progenitor cells from OIP-1 transgenic mouse showed a significant decrease (43%) in osteoclast formation and inhibition (38%) of bone resorption area compared to wild-type mice (5). We determined microarray profiling of differential gene expression in p62 wild-type, non-UBA domain mutation in exon-7 (A381V) and UBA domain mutation in exon-8 (P392L) and MVNP transduced human bone marrow derived preosteoclast cells. We therefore hypothesize that that OIP-1 inhibition of MVNP and mutant p62(P392L) regulated gene expression abrogate pagetic osteoclast development/bone resorption function. Thus, the proposed studies will facilitate development of novel therapeutic agents to control abnormal osteoclastogenesis and high bone turnover in PDB.

Body:

The progress on Task-2 (1-12 months) in the statement of work is as follow:

Task 1. Determine the gene expression profiling with respect to measles virus nucleocapsid (MVNP) and p62 mutant gene expression in pre-osteoclast cells. (Months 1-12): ***Completed***

Task 2. Determine the OIP-1 inhibition of MVNP and p62 mutant regulated gene expression which stimulates osteoclast bone resorption activity. (Months 13-24).—

(a) Examine the osteoclast inhibitory peptide-1 (OIP-1) inhibition of MVNP regulated gene expression in preosteoclast cells (Months 13-16).

Signal regulatory protein beta 1 (SIRPβ1) has been shown to interact with DAP 12, an ITAM containing adaptor protein which plays an important role in osteoclast differentiation. DAP12 and the FcR-gamma chain are required for the normal differentiation and function of osteoclasts in vitro and in vivo (6). Microarray analysis identified that MVNP transduced human bone marrow mononuclear cells demonstrated a high level (353-fold) expression of signal regulatory protein beta 1 (SIRPβ1) (353-fold) compared to empty vector (EV) transduced cells (see abstract copy appended). Real-time PCR analysis of total RNA isolated from normal human peripheral blood monocytes transduced with MVNP confirmed upregulation of SIRPβ1 mRNA expression in the absence of RANKL stimulation. In contrast, RANKL stimulation did not alter SIRPβ1 expression in these cells (Fig.1). To further examine the role of SIRP in DAP12 phosphorylation, peripheral blood mononuclear cells (PBMC) were transduced with EV and MVNP and treated with or without RANKL for 1 hr. Cell lysates were processed for immunoprecipitation with anti-DAP 12 or control IgG antibodies. Western blot analysis of the immune complexes demonstrated an increased ITAM phosphorylation of DAP12 in RANKL stimulated preosteoclast cells either in presence or absence of MVNP, however MVNP stimulation

alone has no effect in the ITAM phosphorylation of DAP12 (Fig.2). We further examined OIP-1 inhibition of FcRIIB gamma chain and DAP12 phosphorylation. Wild-type (WT) and OIP-1 mouse bone marrow derived non-adherent cells were treated with M-CSF and stimulated with or without RANKL (RL) for 1 hr. The total cell lysates were subjected to immunoprecipitation with FcRIIB-gamma-chain specific antibody or DAP12 antibody followed by Western blot analysis using anti-phosphotyrosine (p-Y) antibody. We identified that OIP-1 inhibits the ITIM phosphorylation of FcRIIB-gamma chain but not ITAM phosphorylation of DAP12 (Fig.3).

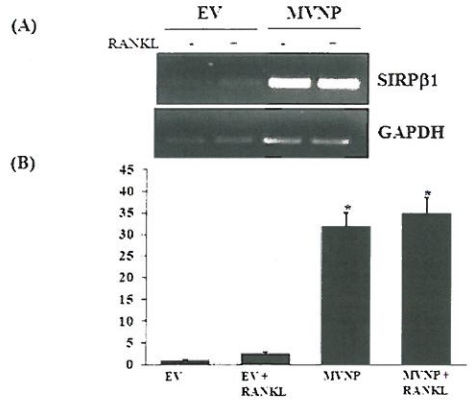


Fig.1 MVNP induces SIPRβ1 expression in human peripheral blood mononuclear cells. (A). Cells were transduced with MVNP retroviral expression plasmid and stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for 48 h. Total RNA isolated was subjected to RT-PCR for SIPRβ1 expression. (B). Total RNA isolated was subjected to real-time PCR for SIPRβ1 expression. The relative level of SIRPβ1 expression was normalized by GAPDH amplification in these cells.

Fig.2 Peripheral blood mononuclear cells (PBMC) were transduced with EV and MVNP and treated with or without RANKL for 60 minutes. Cell lysates were processed for immunoprecipitation with anti-DAP 12 or control IgG antibodies. Immunoprecipitates was Western blot analyzed with anti-DAP12, anti-SIRP-β and anti-phospho G410 (pY) antibodies.

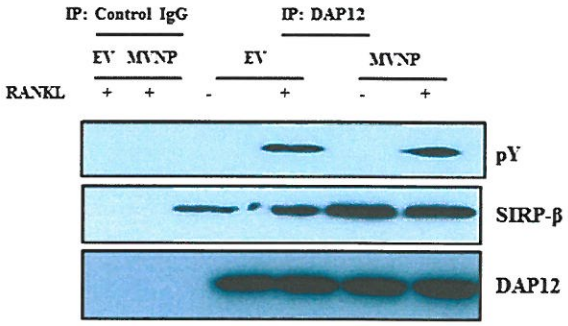
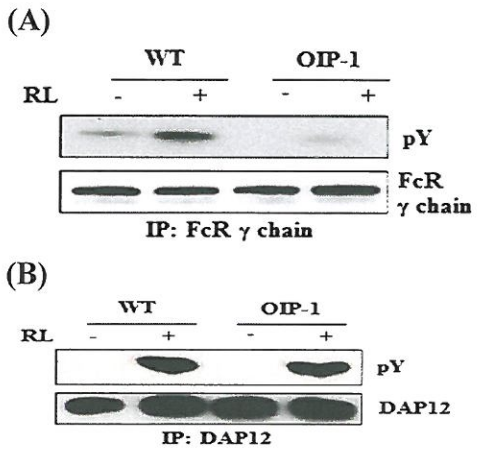


Fig.3 OIP-1 inhibition of FcRIIB-gamma chain ITIM phosphorylation but not DAP12 ITAM phosphorylation. (A) WT and OIP-1 mouse bone marrow derived non-adherent cells were treated with M-CSF and stimulated with or without RANKL (RL) for 60 minutes. Total cell lysates were subjected to immunopreciitation with FcR gamma chain specific antibody followed by Western blotanalysis using anti-phosphotyrosine (p-Y) antibody. (B) Total cell lysates were immunoprecipiated with DAP12 antibody and Westeren blot analyzed using anti-phosphotyrosine (p-Y) antibody.



(b) Determine the OIP-1 suppression of p62 mutant regulated gene expression in preosteoclast cells (Months 17-20).

TRAF6 is an adaptor molecule involved in RANK signaling and polyubiquitination of TRAF6 plays an important role in OCL differentiation (7). To determine whether the $p62^{P392L}$ mutant modulates TRAF6 ubiquitination during OCL differentiation, non-adherent mouse bone marrow cells were transduced with $p62^{WT}$, non-UBA mutant ($p62^{A381V}$) or UBA mutant ($p62^{P392L}$) and stimulated with RANKL (100 ng/ml) and M-CSF (10 ng/ml) for 48 h. Total cell lysates obtained were subjected to immunoprecipitation of TRAF6 using rabbit anti-TRAF6 antibody. Western blot analysis of the immune complexes using anti-ubiquitin antibody revealed accumulation of polyubiquitinated TRAF6 in $p62^{P392L}$ transduced preosteoclast cells with and without RANKL stimulation compared to $p62^{WT}$ and $p62^{A381V}$ transduced cells. However a modest increase in TRAF6 ubiquitination was observed in $p62^{WT}$ and $p62^{A381V}$ compared to control empty vector (EV) transduced preosteoclast cells (Fig.4A). CYLD is a deubiquitinating enzyme which negatively regulates osteoclast differentiation (8). During the previous report period, we showed mutant $p62^{P392L}$ abolished interaction with CYLD and thereby stimulates osteoclast differentiation. Therefore, we next examined if shRNA suppression of CYLD in the presence of $p62^{WT}$ and mutants modulate TRAF6 ubiquitination in preosteoclast cells. Mouse bone marrow-derived non-adherent cells were transduced with $p62^{WT}$, $p62^{A381V}$ or $p62^{P392L}$ in the presence and absence of CYLD shRNA. Cells were stimulated with RANKL (100 ng/ml) and M-CSF (10 ng/ml) for 48 h and total cell lysates obtained were subjected to immunoprecipitation of TRAF6. As shown in Fig.4B, shRNA suppression of CYLD expression significantly increased polyubiquitination of TRAF6 in the presence of $p62^{WT}$, $p62^{A381V}$ and $p62^{P392L}$ compared to non-specific control shRNA transduced preosteoclast cells. These data suggests that $p62^{P392L}$/CYLD modulates TRAF6 ubiquitination during OCL differentiation. We further examined whether $p62^{P392L}$/CYLD modulates downstream effectors of RANK signaling during OCL differentiation. Mouse bone marrow derived non-adherent cells were transduced with $p62^{WT}$, $p62^{A381V}$ or $p62^{P392L}$ and stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for different time points (0-60 min). Western blot analysis of total cell lysates obtained from preosteoclast cells revealed that $p62^{P392L}$ induced high level expression of p-IκB with and without RANKL stimulation compared to $p62^{WT}$ and $p62^{P381V}$ transduced cells. However, we observed a modest increase in p-IκB expression in $p62^{WT}$ and $p62^{A381V}$ compared to EV transduced cells (Fig.5). (Please see the article copy appended).

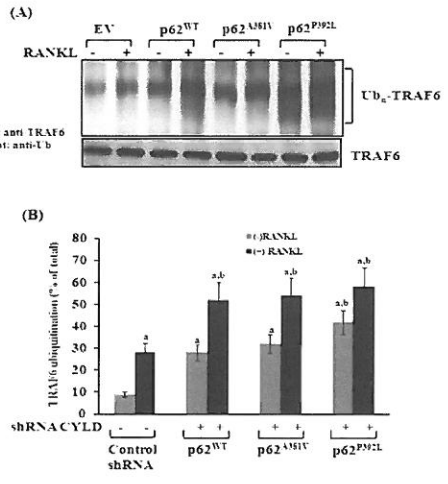


Fig.4. $p62^{P392L}$ enhance TRAF6 polyubiquitination during osteoclast differentiation. (A). Mouse bone marrow derived non-adherent cells were transduced with $p62^{WT}$, $p62^{A381V}$, $p62^{P392L}$ and cells were stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for 48 h. Cells transduced with empty vector (EV) served as control. (B). shRNA knock-down of CYLD expression enhances TRAF6 polyubiquitination (Ub_n) in preosteoclast cells. Cells were transduced retroviral expression plasmids of $p62^{WT}$, $p62^{A381V}$, $p62^{P392L}$ with a non-specific control shRNA and CYLD shRNA and stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for 48 h. Total cell lysates obtained were subjected to immuneprecipitation using anti-TRAF6 antibody and TRAF6 ubiquitination was analyzed by Western blot using rabbit-anti-Ubiquitin antibody. The band intensity was quantified by NIH ImageJ program. The values are expressed as mean ± SD for three independent experiments (^{a,b} $p < 0.05$). ^{a} compared with EV and ^{b} compared with EV stimulated with RANKL.

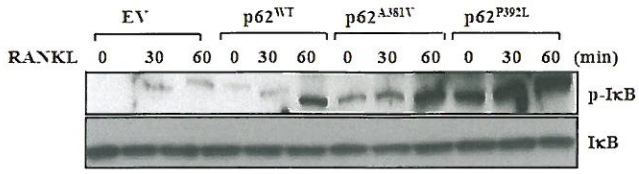


Fig. 5. $p62^{P392L}$ modulation of IκB activation. Mouse bone marrow derived non-adherent cells were transduced with $p62^{WT}$, $p62^{A381V}$, $p62^{P392L}$ and stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for different time points (0-60 min). Cells transduced with empty vector (EV) served as control. Total cell lysates obtained from the preosteoclast cells were analyzed by Western blot for p-IκB expression.

(c) Examine if over-expression of MVNP and P62 mutant regulated genes in OIP-1 mice derived preosteoclasts induce pagetic phenotype in osteoclasts (Months 20-24).

Pagetic osteoclasts produce increased levels of IL-6 and express high levels of IL-6 receptors compared to normal subjects (2). We next examined the IL6 gene expression in the MVNP stimulated preosteoclast cells. Mouse bone marrow derived non-adherent cells were transduced with a control empty vector (EV) or MVNP and treated with and without RANKL for 24 hr. Quantitative real-time PCR analysis of total RNA isolated from these cells demonstrated a significant increase in MVNP and RANKL stimulated with MVNP transduced preosteoclast cells (Fig.6). To determine the OIP-1 inhibition of IL-6 production, wild-type (WT) and OIP-1 mouse bone marrow cells were transduced with EV or MVNP and cultured in the presence or absence of M-CSF and RANKL for 5 days. Condition media were analyzed for IL-6 levels by ELISA. We identified that MVNP significantly increased IL-6 production and was further potentiated by RANKL stimulation when compared to condition media collected from EV stimulated osteoclast cells. We found OIP-1 significantly inhibits IL6 production in both MVNP transduced and RANKL stimulated osteoclast cells (Fig.7).

Fig.6. MVNP modulation of IL-6 expression. Mouse bone marrow derived non-adherent cells were transduced with EV or MVNP and treated with and without RANKL for 24 hrs. Total RNA isolated was subjected to quantitative real-time PCR analysis for IL-6 expression. Relative levels of IL-6 gene expression were normalized with respect to the levels of β-actin amplification. Values expressed as mean±SE for three independent experiments.

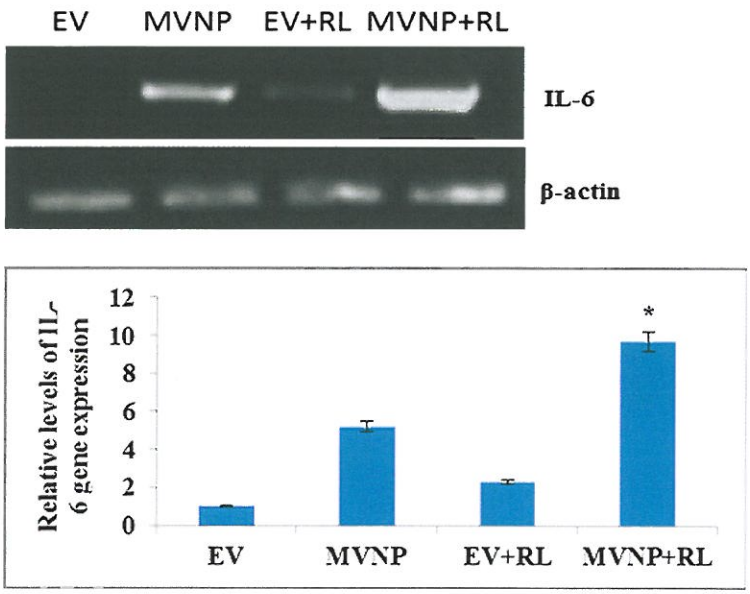
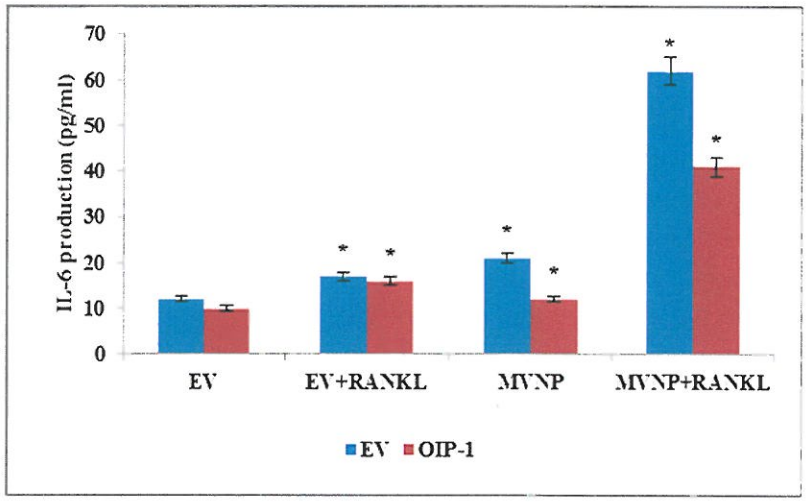
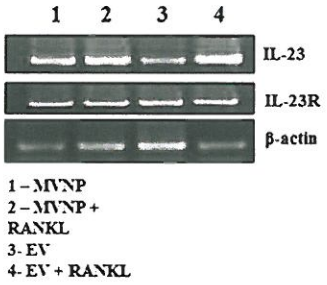


Fig.7. OIP-1 inhibition of IL-6 expression. (A) Wild-type (WT) and OIP-1 mouse bone marrow cells were transduced with EV or MVNP and cultured in the presence or absence of M-CSF (10 ng/ml) and RANKL (100 ng/ml) for 5 days. Condition media were harvested on 5th day and the concentrated. The concentration of IL-6 was determined using ELISA. Results are reported as IL-6 concentration (pg/ml) and are the mean±SD of triplicate samples.



IL-23 and IL-27 are novel cytokine belonging to the IL-6/IL-12 cytokine family. Therefore, we studied the levels of IL-23 in pagetic pre-osteoclast cells. We transduced with EV or MVNP in non-adherent cells from peripheral blood mononuclear cells (PBMC) and stimulated with or without RANKL for 48 hr. Quantitative real-time PCR analysis of total RNA isolated from these cells demonstrated increased levels of IL-23 expression in the MVNP transduced cells. Also, RANKL treatment further increased IL-23 levels in both EV and MVNP transduced cells. We also identified IL-23 receptor expressed in preosteoclast cells, however no significant change in MVNP transduced or in RANKL treated EV or MVNP transduced cells (Fig.8). We confirm IL-23 receptor is expression in the mature osteoclast by confocal microscopy analysis (Fig.9). Further experiments are underway to examine OIP-1 inhibition of IL-23 and IL-23 receptor expression and osteoclast differentiation/bone resorption.

Fig.8. MVNP regulation of IL-23 and IL-23 receptor expression during osteoclast differentiation. PBMC transduced with EV or MVNP and stimulated with or without RANKL for 48 hr. Total RNA isolated was subjected to quantitative real-time PCR analysis for IL-23 expression. Relative levels of IL-23 gene expression were normalized with respect to the levels of β-actin amplification. Values expressed as mean±SE for three independent experiments.



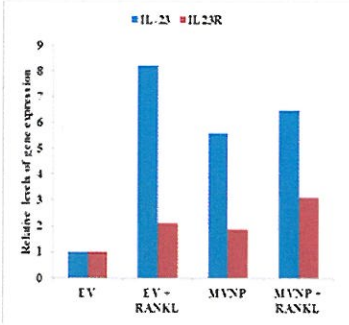
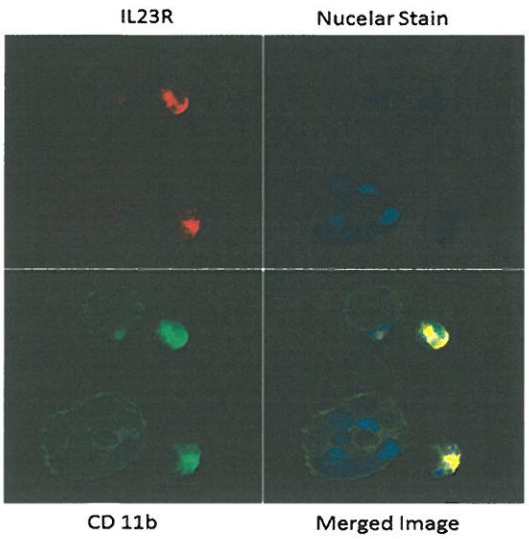


Fig.9. IL-23 receptor expression in osteoclast (OCL) formed from PBMC cultures. PBMC derived from normal were cultured to form OCL in the presence of 10 ng/mL hM-CSF, 1 μm dexamethasone and 100 ng/mL hRANKL. The cultures were fixed and processed for confocal image analysis using anti-IL23 receptor antibody and CD11b antibody. The merged image demonstrated the expression of IL23 on the osteoclast membrane.



Task 3. Assess the potential of OIP-1 to inhibit MVNP and p62 mutant induced osteotropic cytokines such as IL-1, IL-6, FGF-2 production by osteoclasts and stimulation of RANKL gene expression in bone microenvironment. (Months 25-36). -- ***Not yet initiated***

Key Research Accomplishments:

- We identified measles virus nucleocapsid (MVNP) induce high level expression of signal regulatory protein beta 1 (SIRPβ1) which interact with DAP 12, an ITAM containing adaptor protein which plays an important role in osteoclast differentiation.
- We demonstrated that mutant $p62^{P392L}$ expression results in accumulation of polyubiquitinated TNF receptor-associated factor, TRAF6 and elevated levels of phospho-IκB during osteoclast differentiation.

Reportable Outcomes

Article(s):

Sundaram K, Shanmugarajan S, Rao DS and **Reddy SV**. Mutant $p62^{P392L}$ stimulation of osteoclast differentiation in Paget's disease of bone. Endocrinology, 2011 (In press).

Abstract(s):

Sundaram K, Shanmugarajan S, Rao DS and **Reddy SV**. Microarray profile of $p62^{P392L}$ and measles virus nucleocapsid protein (MVNP) regulated gene expression during osteoclast differentiation. ASBMR 32st Annual meeting, Sept. 2011, San Diego, CA.

Conclusions

In conclusion, we showed MVNP induce signal regulatory protein beta 1 (SIRPβ1) during osteoclast differentiation. Furthermore, $p62^{P392L}$ expression results in accumulation of polyubiquitinated TRAF6 and elevated levels of p-IκB during OCL differentiation. Thus, $p62^{P392L}$ mutant and MVNP regulated gene expression during osteoclast differentiation provides new insights into the molecular mechanisms and therapeutic targets to control elevated osteoclast activity in PDB

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Appendices

Copy of the article noted under reportable outcomes is appended.

[FR0164] Microarray Profile of $p62^{P392L}$ and Measles Virus Nucleocapsid Protein (MVNP) Regulated Gene Expression During Osteoclast Differentiation

Kumaran Sundaram, Charles P. Darby Children's Research Institute, USA; Srinivasan Shanmugarajan, Charles P. Darby Children's Research Institute, USA; Sudhaker Rao, Henry Ford Hospital, USA; Sakamuri Reddy, Charles P. Darby Children's Research Institute, USA.

Paget's disease of bone (PDB) is a chronic localized bone disorder affecting 2-3% of the population over 60 years of age. PDB is inherited as an autosomal dominant trait with genetic heterogeneity. SQSTM1/p62 UBA domain mutation ($p62^{P392L}$) is widely identified in PDB and has been shown to increase osteoclastogenesis. Further, environmental factors such as paramyxovirus are implicated in PDB and MVNP has been shown to induce Pagetic phenotype in osteoclasts. However, the molecular mechanisms underlying $p62^{P392L}$ and MVNP stimulation of osteoclast differentiation in PDB are unclear. We therefore determined $p62^{P392L}$ and MVNP regulated gene expression profiling during osteoclast differentiation. Total RNA isolated from normal human bone marrow mononuclear cells transduced with $p62^{WT}$, $p62^{P392L}$, MVNP retroviral expression vectors and stimulated with M-CSF and RANKL for 48 h were subjected to Agilent microarray (~26,000 genes) analysis. We identified 9.7% and 8.4% of genes were upregulated (> 4-fold) in $p62^{P392L}$ and MVNP transduced cells respectively. $P62^{P392L}$ mutant increased Integrin β3 (185 fold), integrin β5 (26 fold), IL-1α (11 fold), IL-6R (8 fold), CXCL-2 (7.5 fold), CXCL-3 (5 fold) compared to $p62^{WT}$ transduced cells. Similarly, MVNP increased integrin β3 (63 fold), NFAT activating protein (6.5 fold), OSCAR (5.5 fold), TRAF5 (8.5 fold) mRNA expression compared to empty vector (EV) transduced cells. MVNP also elevated gene expression of cytokines/growth factors such as IL-17 (18 fold), IL-1F7 (10 fold), IL-17R (4.5 fold) and IL-11 (7 fold). Interestingly, MVNP transduced cells demonstrated a high level expression of signal regulatory protein beta 1 (SIRPβ1) (353 fold). SIRPβ1 has been shown to interact with DAP 12, an ITAM containing adaptor protein which plays an important role in osteoclast differentiation. Real-time PCR analysis of total RNA isolated from normal human peripheral blood monocytes transduced with MVNP confirmed upregulation of SIRPβ1 mRNA expression in the absence of RANKL stimulation. In contrast, RANKL stimulation did not alter SIRPβ1 expression in these cells. Furthermore, bone marrow mononuclear cells derived from patients with PDB showed high levels of SIRPβ1 mRNA expression compared to normal subjects. Thus, $p62^{P392L}$ mutant and MVNP regulated gene expression profiling during osteoclast differentiation provides new insights into the molecular mechanisms and therapeutic targets to control elevated osteoclast activity in PDB.

Disclosure(s): Kumaran Sundaram has nothing to disclose.

Date: Friday, September 16, 2011
Session Info: Plenary Sessions: Welcome Reception & Plenary Poster Session (5:45 PM-7:00 PM)
Presentation Time: 5:45 pm
Room: Hall GH - San Diego Convention Center

Close Window

Mutant p62^{P392L} Stimulation of Osteoclast Differentiation in Paget's Disease of Bone

Kumaran Sundaram, Srinivasan Shanmugarajan, D. Sudhaker Rao, and Sakamuri V. Reddy

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Paget's disease of the bone (PDB) is an autosomal dominant trait with genetic heterogeneity, characterized by abnormal osteoclastogenesis. Sequestosome 1 (p62) is a scaffold protein that plays an important role in receptor activator of nuclear factor κB (RANK) signaling essential for osteoclast (OCL) differentiation. p62^{P392L} mutation in the ubiquitin-associated (UBA) domain is widely associated with PDB; however, the mechanisms by which p62^{P392L} stimulate OCL differentiation in PDB are not completely understood. Deubiquitinating enzyme cylindromatosis (CYLD) has been shown to negatively regulate RANK ligand-RANK signaling essential for OCL differentiation. Here, we report that CYLD binds with the p62 wild-type (p62^{WT}), non-UBA mutant (p62^{A381V}) but not with the UBA mutant (p62^{P392L}) in OCL progenitor cells. Also, p62^{P392L} induces expression of c-Fos (2.8-fold) and nuclear factor of activated T cells c1 (6.0-fold) transcription factors critical for OCL differentiation. Furthermore, p62^{P392L} expression results in accumulation of polyubiquitinated TNF receptor-associated factor (TRAF)6 and elevated levels of phospho-IκB during OCL differentiation. Retroviral transduction of p62^{P392L}/CYLD short hairpin RNA significantly increased TRAP positive multinucleated OCL formation/bone resorption activity in mouse bone marrow cultures. Thus, the p62^{P392L} mutation abolished CYLD interaction and enhanced OCL development/bone resorption activity in PDB. (***Endocrinology* 152: 0000–0000, 2011**)

Paget's disease of bone (PDB) is a chronic focal skeletal disorder that affects 2–3% of the population over the age of 60 yr. PDB is inherited as an autosomal dominant trait with genetic heterogeneity and characterized by highly localized areas of bone turnover with increased osteoclast (OCL) activity followed by an exaggerated osteoblast response (1). OCL in PDB contain paramyxoviral-nuclear inclusions and nucleocapsid transcripts (2). The nuclear inclusions were present in 20–40% of OCL in all patients with PDB (3). Furthermore, expressions of measles virus nucleocapsid (MVNP) transcripts have been detected in approximately 80% of bone marrow samples from patients with PDB (4). Recently, Merchant *et al.* (5) have found MVNP expression in pagetic bone and pagetic osteosarcomas. Previously, canine distemper virus nucleocapsid transcripts were also detected in pagetic bone samples (6). However, other workers have been unable to detect expression of paramyxoviral transcripts in PDB (7, 8). Sequestosome 1 (p62) (also known as sequestosome 1) is a multifunctional ubiquitin-binding protein, which plays an important role in cell signaling, receptor internalization, and protein turnover (9). Recurrent mutations in p62 ubiquitin-associated (UBA) domain have been identified in about 25% of familial and less than 10% of sporadic cases; however, the P392L amino acid substitution being the most common in PDB (10). Furthermore, a mutation in p62 exon-7 (K378X), which introduces a premature stop codon and thus eliminates entire UBA domain, has been reported (11). Najat *et al.* (12) have also identified a non-UBA domain mutation, p62^{A381V} associated with

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Abbreviations: AP-1, Activator protein 1; CYLD, cylindromatosis; EV, empty vector; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; K63, lysine-63; M-CSF, macrophage colony-stimulating factor; MVNP, measles virus nucleocapsid; NFATc1, nuclear factor of activated T cells c1; NF-κB, nuclear factor κB; OCL, osteoclast; p62, sequestosome 1; PDB, Paget's disease of bone; RANK, receptor activator of NF-κB; RANKL, RANK ligand; shRNA, short hairpin RNA; TRAF, TNF receptor-associated factor; TRAP, tartrate-resistant acid phosphatase; UBA, ubiquitin associated.

PDB. p62 has been shown to physically associate with TNF receptor-associated factor (TRAF)6 and involved in receptor activator of nuclear factor κB (NF-κB) (RANK) signaling critical for OCL differentiation (13). p62 is a scaffold protein that mediates RANK ligand (RANKL) signaling by activating transcription factors such as NF-κB and activator protein 1 (AP-1), which induces the nuclear factor of activated T cells (NFATc1) transcription factor expression essential for osteoclastogenesis (14–16). p62 knockout mice have impaired osteoclastogenesis in response to parathyroid hormone-related peptide (13). It has been shown that p62 mutant (p62^{P392L}) transgenic mice have increased OCL formation but do not develop focal osteolytic lesions with the characteristics of PDB (17). In contrast, recently, p62 UBA domain mutation (p62^{P394L}) has been shown to be sufficient to cause a Paget's disease-like disorder in mice (18). Also, truncation of the p62 UBA domain causes aberrant RANK signaling and increased osteoclastogenesis in RAW 264.7 cell cultures (19). Recently, p62^{P392L} mutation has been shown to alter RANKL signaling and induces activation of human OCL (20). However, the molecular mechanisms by which p62^{P392L} stimulates OCL differentiation in PDB are not completely understood.

Pridgeon *et al.* (21), using an *in vitro* expression cloning approach, identified several proteins that interact with the p62 UBA domain. The tumor suppressor cylindromatosis (CYLD) gene was first identified in human affected with familial CYLD, a genetic syndrome in which numerous benign tumors of skin develop, principally on the head and neck region. The disease is inherited in an autosomal manner and is caused by germline mutations in the CYLD gene on chromosome 16q12-q13, which predicts truncation or absence of the encoded protein (22). CYLD protein has been shown to physically interact with p62, and it negatively regulates osteoclastogenesis (23). CYLD is a deubiquitinase that removes the ubiquitin chain from several proteins, particularly TRAF2, TRAF6, and NF-κB essential modulator, and inactivates NF-κB signaling (24–26). The deubiquitination activity of CYLD is highly specific for proteins at lysine-63 (K63)-linked ubiquitin chains in substrate but has been shown to act on K48-linked polyubiquitin chains (27). Polyubiquitination of target protein at K63 influences protein-protein interactions, which play an important role in cell signaling, and ubiquitination at K48 directs to proteosomal degradation via ubiquitin-proteosomal pathway (28). Here, we report that the p62 UBA mutant (p62^{P392L}) abolished the interaction with CYLD, which implicates a potential role in enhanced OCL development in PDB.

Materials and Methods

Reagents and antibodies

Cell culture and DNA transfection reagents were purchased from Invitrogen Corp. (Carlsbad, CA). RANKL and macrophage colony-stimulating factor (M-CSF) were obtained from R&D Systems, Inc. (Minneapolis, MN). Rabbit-anti-CYLD antibody was purchased from Abcam (Cambridge, MA). Rabbit-anti-NFATc1, anti-c-Fos, antihemagglutinin (HA) tag, and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). pNFAT, pNF-κB, and pAP-1-Luc cis-reporter plasmids were obtained from Stratagene (La Jolla, CA). SuperSignal enhanced chemiluminescence reagent was obtained from Amersham Bioscience (Piscataway, NJ), and nitrocellulose membranes were purchased from Millipore (Bedford, MA). A luciferase reporter assay system was obtained from Promega (Madison, WI).

p62 mutagenesis

A plasmid (pcDNA3-HA-p62) containing the full-length human *p62* cDNA (>85% homologous to murine p62) was kindly provided by Jorge Moscat (Sanford-Burnham Medical Research Institute, La Jolla, CA), and the P392L mutation in UBA domain (exon-8) and A381V in non-UBA domain (exon-7) were introduced by site-directed mutagenesis using QuikChange II XL Site-Directed Mutagenesis kit (Stratagene, Inc., La Jolla, CA) as described earlier (29). The mutagenized p62 cDNA was sequence verified for correct introduction of P392L and A381V mutations (Fig. 1A).

p62 and CYLD short hairpin RNA (shRNA) retroviral expression

The p62^{WT} and mutant cDNA were excised from the pcDNA3-HA-p62 plasmids by digestion with *Eco*RI and subcloned into the pLXSN retroviral vector (CLONTECH Laboratories, Inc., Palo Alto, CA). The resulting plasmid construct transcribes p62 mRNA expression under the control of 5′ long terminal repeat viral promoter elements. The recombinant p62 constructs and CYLD shRNA retroviral plasmid (Open Biosystems, Rockford, IL) were transfected into the PT67 amphotropic packaging cell line using lipofectamine (Invitrogen Corp.). Stable clonal cell lines producing p62 recombinant retrovirus at high titer (1×10^6 virus particles/ml) were established by selecting for resistance to neomycin (600 μg/ml). Similarly, a control retrovirus producer cell line was established by transfecting the cells with the pLXSN empty vector (EV). Producer cell lines were maintained in DMEM containing 10% fetal calf serum (FCS), 100 U/ml each of streptomycin and penicillin, 4 mM L-glutamine, and high glucose (4.5 g/liter). Retroviral supernatants from the producer cell cultures were collected and filtered (0.45 μm pore diameter) for immediate use. Mouse bone marrow-derived nonadherent cells were transduced with p62^{WT}, p62^{P392L}, or p62^{A381V} and CYLD shRNA retroviral supernatants (20%) from the producer cell lines in the presence of polybrene (4 μg/ml) for 24 h at 37 C in a 5% CO_2 incubator as described earlier (30).

OCL culture and bone resorption assay

Mouse bone marrow-derived nonadherent cells were dispersed into α-MEM containing 10% FCS and were seeded in

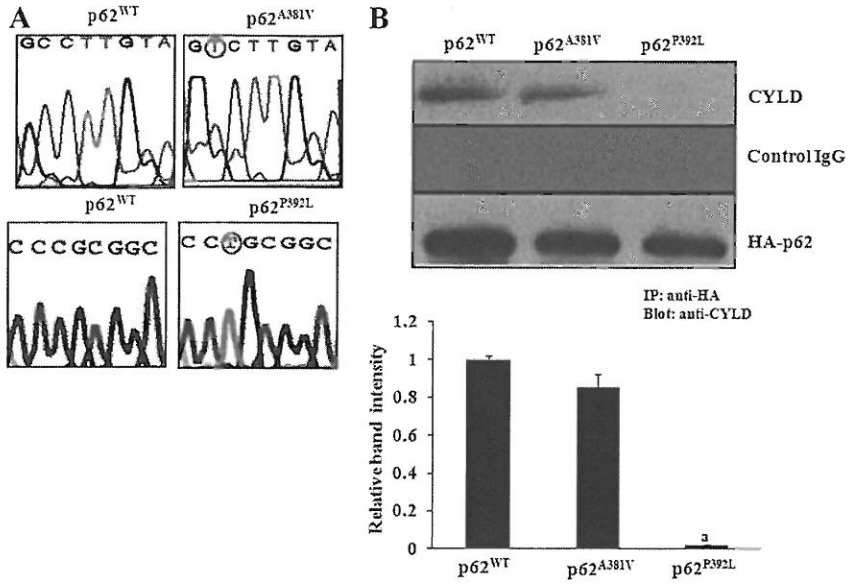

FIG. 1. p62 interaction with CYLD. A, Site-directed mutagenesis of p62 non-UBA ($p62^{A381V}$) and UBA ($p62^{P392L}$) mutations associated with PDB. A plasmid containing the full-length human p62 cDNA was used as template for site-directed mutagenesis of UBA mutant, $p62^{P392L}$ (C-to-T transition), and a non-UBA mutation, $p62^{A381V}$ (C-to-T transition), as described in *Materials and Methods*. The mutagenized p62 cDNA sequenced to verify correct introduction of the P392L, A381V mutations as *circled*. B, p62 UBA mutation ($p62^{P392L}$) abolished interaction with CYLD in pre-OCL cells. Mouse bone marrow-derived nonadherent cells were transduced with retroviral expression plasmids of $p62^{WT}$, $p62^{A381V}$, or $p62^{P392L}$, and cells were stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for 48 h. Total cell lysates obtained were subjected to immunoprecipitation using rabbit anti-HA antibody. The immunoprecipitants were analyzed by Western blotting using rabbit anti-CYLD antibody. The band intensity was quantified by National Institutes of Health ImageJ program, and CYLD immunoprecipitation was normalized with HA-tagged p62 expressed in these cells. The values are expressed as mean ± SD for three independent experiments (a, $P < 0.05$).

96-well plates at 6×10^5 cells/well in 0.2 ml of medium. Cells were transduced with $p62^{WT}$, $p62^{A318V}$, or $p62^{P392L}$ and cultured in the presence of RANKL (100 ng/ml) and M-CSF (10 ng/ml) and were refed twice weekly by semidepletion (half of the medium withdrawn and replaced with fresh medium). At the end of culture period (5 d), the cells were fixed with 2% glutaraldehyde in PBS and stained for tartrate-resistant acid phosphatase (TRAP) activity using a histochemical kit (Sigma, St. Louis, MO). TRAP positive multinucleated cells containing three or more nuclei were scored as OCL cells under a microscope. Bone resorption activity of the OCL was assayed by culturing OCL for 10 d on dentine slices. At the end of the culture period, adherent cells were removed from the dentine disc, using 1 M NaOH and stained with 0.1% toluidine blue. The bone resorption area was quantified using computerized image analysis (Adobe Photoshop and Scion MicroImaging version 4.2). The percentage of the resorbed area was calculated relative to the total dentine disc area.

Coimmunoprecipitation assay

Mouse bone marrow-derived nonadherent cells were seeded in six-well plates (5×10^5 cells/well) were transduced with $p62^{WT}$, $p62^{P392L}$, or $p62^{A381V}$ retroviral expression vectors and stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for 48 h. Total cell lysates were collected in a lysis buffer [50 mM HEPES (pH 7.5), 250 mM NaCl, 0.2 mM EDTA, 10 μM NaF, and 0.5% Nonidet P-40] were immunoprecipitated using anti-HA antibody as described earlier (29). Immunocomplexes were subjected to Western blot analysis for CYLD using rabbit anti-CYLD antibody.

Quantitative real-time RT-PCR

NFATc1 mRNA expression was measured by real-time RT-PCR as described previously (29). Briefly, total RNA was isolated from pre-OCL cells transfected with $p62^{WT}$, $p62^{A381V}$, or $p62^{P392L}$ and were stimulated with and without RANKL (100 ng/ml) for 48 h, using RNAzol reagent (Biotecx Laboratories, Houston, TX). A RT reaction was performed using a cDNA synthesis kit (Bio-Rad, Hercules, CA) in a 25-μl reaction volume containing total RNA (2 μg), 1× PCR buffer, and 2 mM $MgCl_2$, at 42 C for 15 min followed by 95 C for 5 min. The quantitative real-time PCR was performed using IQ SYBR Green Supermix in an iCycler (iCycler iQ Single-color Real Time PCR Detection System; Bio-Rad). The primer sequences used to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were 5′-CCTA CCCCCAATGTATCCGTTGTG-3′ (sense) and 5′-GGAGGAATGGGAGTTGCTGTT GAA-3′ (antisense) and for mouse NFATc1 mRNA were 5′-GGCCGCAGAACACTAC AGTTA-3′ (sense) and 5′-GAGATACCCG GGGTGGAC-3′ (antisense). Thermal cycling parameters were 94 C for 3 min, followed by 40 cycles of amplifications at 94 C for 30 sec, 60 C for 1 min, 72 C for 1 min, and 72 C for 5 min as the final elongation step. Relative levels of NFATc1 mRNA expression were normalized in all the samples analyzed with respect to GAPDH amplification.

NF-κB, AP-1, and NFAT-Luc reporter gene assay

RAW 264.7 cells were cultured in DMEM supplemented with 10% FCS in a humidified atmosphere with 5% CO_2 at 37 C. DNA transfections were performed using lipofectamine transfection reagent (Invitrogen Corp.) according to the manufacturer's protocol. RAW 264.7 cells were transfected with NF-κB, AP-1, and NFAT-Luc reporter plasmids and coexpressed with $p62^{WT}$, $p62^{P392L}$, or $p62^{A381V}$. Cells were cultured in the presence or absence of RANKL (100 ng/ml) for 48 h. The cell monolayer was washed twice with PBS and incubated at room temperature for 15 min with 0.3 ml cell lysis buffer. The monolayer was scraped and spun briefly in a microfuge to pellet the debris. Then, a 20-μl aliquot of each sample was mixed with 100 μl of the luciferase assay reagent. Light emission was measured for 10 sec of integrated time using Sirius Luminometer (Promega). The transfection efficiency was normalized by cotransfection with 0.2 μg of pRSV β-gal plasmid and measuring β-galactosidase activity in the cell lysates. LacZ cytochemical activity staining (Invitrogen Corp.) indicated a DNA transfection efficiency (>80%) in RAW 264.7 cells.

TRAF6 ubiquitination assay

Mouse bone marrow-derived nonadherent cells were seeded in six-well plates (5×10^5 cells/well), transduced with $p62^{WT}$,

$p62^{P392L}$, or $p62^{A381V}$ retroviral expression vectors, and stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml). After 48 h, total cell lysates were collected in a lysis buffer [50 mM HEPES (pH 7.5), 250 mM NaCl, 0.2 mM EDTA, 10 μM NaF, and 0.5% Nonidet P-40]. TRAF6 was immunoprecipitated using rabbit-anti-TRAF6 antibody, and the ubiquitin-conjugated TRAF6 was detected by Western blotting using rabbit-antiubiquitin antibody.

Statistical analysis

Results are presented as mean ± SD for three independent experiments and were compared by Student's *t* test. Values were considered significantly different for a and b, $P < 0.05$.

Results

p62 UBA mutation ($p62^{P392L}$) abolished interaction with CYLD in pre-OCL cells

To examine the functional role of $p62^{P392L}$ in OCL differentiation, we developed HA-tagged $p62^{WT}$, non-UBA mutant ($p62^{A381V}$), and UBA mutant ($p62^{P392L}$) retroviral expression vectors as described in *Materials and Methods*. Mouse bone marrow-derived nonadherent cells were transduced with $p62^{WT}$, $p62^{A381V}$, or $p62^{P392L}$ expression vectors and stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for 48 h. p62 was then immunoprecipitated from the total cell lysates using anti-HA antibody. Western blot analysis of the immunocomplex revealed that CYLD coimmunoprecipitated with $p62^{WT}$ and non-UBA mutant $p62^{A381V}$ but not with UBA mutant $p62^{P392L}$. In contrast, a control nonspecific rabbit IgG did not immunoprecipitate CYLD (Fig. 1B). These results suggest that the p62 UBA mutation ($p62^{P392L}$) abolished interaction with CYLD in pre-OCL cells.

$p62^{P392L}$ enhances TRAF6 ubiquitination during OCL differentiation

TRAF6 is an adaptor molecule involved in RANK signaling, and polyubiquitination of TRAF6 plays an important role in OCL differentiation. To determine whether the $p62^{P392L}$ mutant modulates TRAF6 ubiquitination during OCL differentiation, nonadherent mouse bone marrow cells were transduced with $p62^{WT}$, non-UBA mutant ($p62^{A381V}$), or UBA mutant ($p62^{P392L}$) and stimulated with RANKL (100 ng/ml) and M-CSF (10 ng/ml) for 48 h. Total cell lysates obtained were subjected to immunoprecipitation of TRAF6 using rabbit anti-TRAF6 antibody. Western blot analysis of the immunocomplexes using antiubiquitin antibody revealed accumulation of polyubiquitinated TRAF6 in $p62^{P392L}$ transduced pre-OCL cells with and without RANKL stimulation compared with $p62^{WT}$ and $p62^{A381V}$ transduced cells. However, a modest increase in TRAF6 ubiquitination was observed in $p62^{WT}$

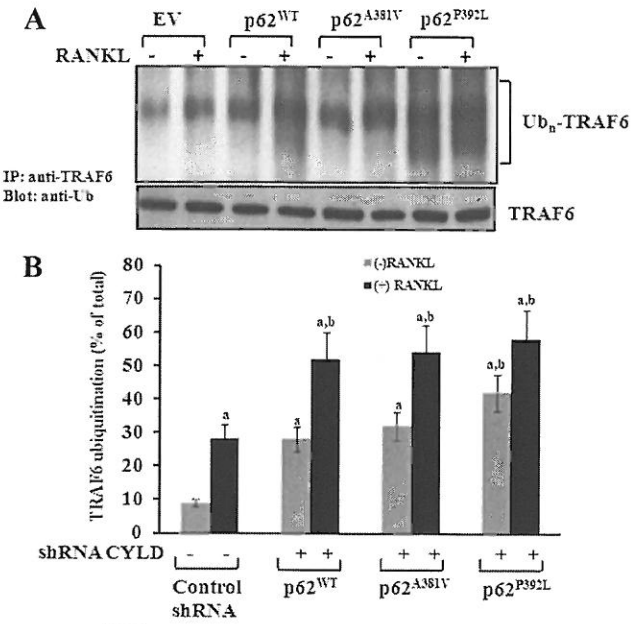

FIG. 2. $p62^{P392L}$ enhance TRAF6 polyubiquitination during OCL differentiation. A, Mouse bone marrow-derived nonadherent cells were transduced with $p62^{WT}$, $p62^{A381V}$, or $p62^{P392L}$, and cells were stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for 48 h. Cells transduced with EV served as control. B, shRNA knockdown of CYLD expression enhances TRAF6 polyubiquitination (Ub_n) in pre-OCL cells. Cells were transduced retroviral expression plasmids of $p62^{WT}$, $p62^{A381V}$, or $p62^{P392L}$ with a nonspecific control shRNA and CYLD shRNA and stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for 48 h. Total cell lysates obtained were subjected to immunoprecipitation using anti-TRAF6 antibody, and TRAF6 ubiquitination was analyzed by Western blotting using rabbit-antiubiquitin antibody. The band intensity was quantified by National Institutes of Health ImageJ program. The values are expressed as mean ± SD for three independent experiments (a and b, $P < 0.05$; compared with EV with and without RANKL stimulation, respectively).

and $p62^{A381V}$ compared with control EV-transduced pre-OCL cells (Fig. 2A). We next examined whether shRNA suppression of CYLD in the presence of $p62^{WT}$ and mutants modulate TRAF6 ubiquitination in pre-OCL cells. Mouse bone marrow-derived nonadherent cells were transduced with p62 ^{WT}, $p62^{A381V}$, or $p62^{P392L}$ in the presence and absence of CYLD shRNA. Cells were stimulated with RANKL (100 ng/ml) and M-CSF (10 ng/ml) for 48 h, and total cell lysates obtained were subjected to immunoprecipitation of TRAF6. As shown in Fig. 2B, shRNA suppression of CYLD expression significantly increased polyubiquitination of TRAF6 in the presence of $p62^{WT}$, $p62^{A381V}$, and $p62^{P392L}$ compared with nonspecific control shRNA transduced pre-OCL cells. These data suggests that $p62^{P392L}$/CYLD modulates TRAF6 ubiquitination during OCL differentiation.

$p62^{P392L}$ modulation of downstream effectors of RANK signaling

$p62^{P392L}$ has been shown to increase OCL differentiation (17, 20). Therefore, we examined whether $p62^{P392L}$/

CYLD modulates downstream effectors of RANK signaling during OCL differentiation. Mouse bone marrow-derived nonadherent cells were transduced with $p62^{WT}$, $p62^{A381V}$, or $p62^{P392L}$ and stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for different time points (0–60 min). Western blot analysis of total cell lysates obtained from pre-OCL cells revealed that $p62^{P392L}$ induced high level expression of phospho-IκB with and without RANKL stimulation compared with $p62^{WT}$ and $p62^{P381V}$ transduced cells. However, we observed a modest increase in p-IκB expression in $p62^{WT}$ and $p62^{A381V}$ compared with EV transduced cells (Fig. 3A). Furthermore, pre-OCL cells stimulated with RANKL and M-CSF for 48 h had increased (2.8-fold) c-Fos expression in $p62^{P392L}$ compared with $p62^{WT}$ or $p62^{A381V}$ transduced cells (Fig. 3B). To further examine the role of $p62^{P392L}$/CYLD on c-Fos expression, mouse bone marrow-derived nonadherent cells were transduced with CYLD shRNA coexpressed with $p62^{WT}$, $p62^{A381V}$, or $p62^{P392L}$ and stimulated with RANKL and M-CSF for 48 h. Western blot analysis of total cell lysates identified that CYLD knockdown significantly increased c-Fos expression in $p62^{WT}$, $p62^{A381V}$, or $p62^{P392L}$ mutant transduced cells without RANKL stimulation compared with control shRNA transduced cells (Fig. 3C). shRNA suppression (~80%) of CYLD expression was confirmed by Western blot analysis (Fig. 3D).

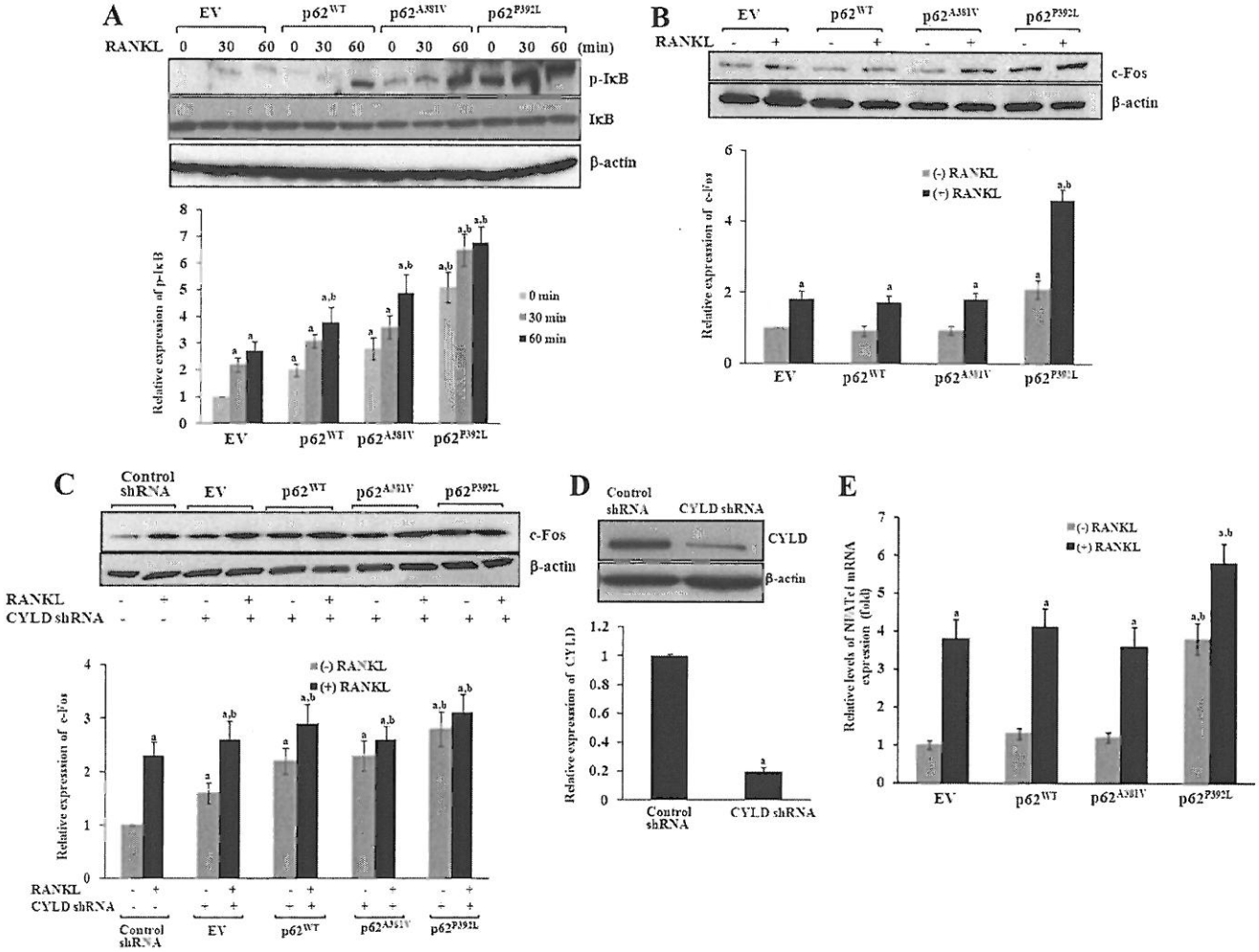

FIG. 3. $p62^{P392L}$ modulation of RANK signaling. Mouse bone marrow-derived nonadherent cells were transduced with $p62^{WT}$, $p62^{A381V}$, or $p62^{P392L}$ and stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for different time points (0–60 min). Cells transduced with EV served as control. A, Total cell lysates obtained from the pre-OCL cells were analyzed by Western blotting for p-IκB expression. B, Total cell lysates obtained from pre-OCL cells stimulated with M-CSF and RANKL for 48 h were analyzed by Western blotting for c-Fos expression. C, Mouse bone marrow-derived nonadherent cells were transduced with $p62^{WT}$, $p62^{A381V}$, or $p62^{P392L}$ with presence of CYLD shRNA. Cells transduced with a nonspecific shRNA served as control. Cells were stimulated with M-CSF (10 ng/ml) and RANKL (100 ng/ml) for 48 h, and total cell lysates obtained were subjected to Western blot analysis of c-Fos expression. D, Western blot analysis of shRNA suppression of CYLD expression compared with nonspecific control shRNA. E, Total RNA isolated from pre-OCL cells transduced with $p62^{WT}$, $p62^{A381V}$, or $p62^{P392L}$ and stimulated with RANKL and M-CSF for 48 h was subjected to real-time PCR analysis for NFATc1 mRNA expression and normalized with GAPDH mRNA amplification in these cells. The band intensity was quantified by National Institutes of Health ImageJ program, and values are expressed as mean ± SD for three independent experiments (a and, b $P < 0.05$; compared with EV with and without RANKL stimulation, respectively).

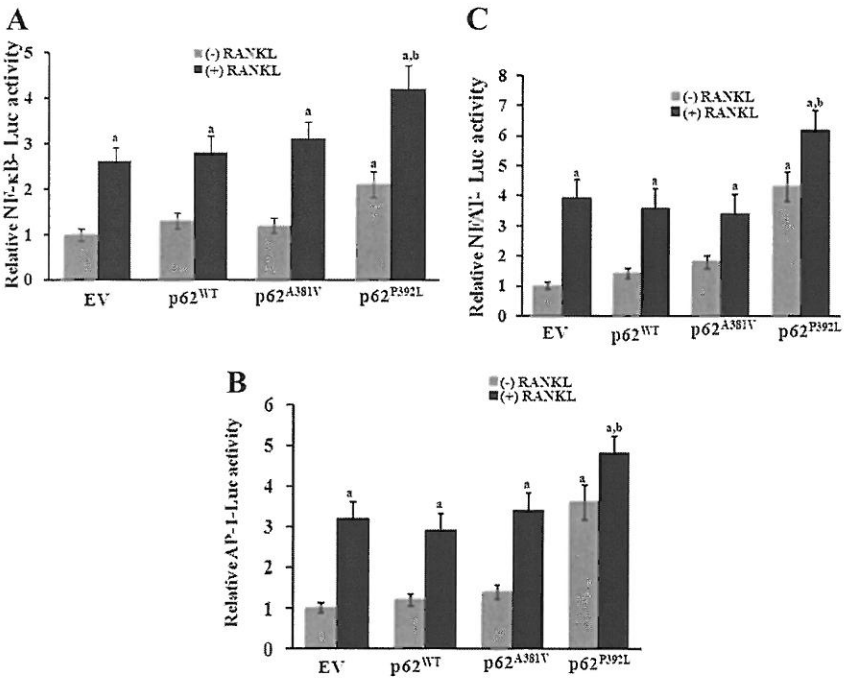

FIG. 4. A, p62^{P392L} enhances NF-κB, AP-1 (B), and NFAT-Luc reporter gene activity (C). RAW 264.7 cells were transfected with NF-κB, AP-1, and NFAT- Luc reporter plasmids and coexpressed with p62^{WT}, p62^{A381V}, or p62^{P392} and stimulated with and without RANKL (100 ng/ml) for 48 h. Total cell lysates prepared were assayed for luciferase activity. The transfection efficiency was normalized by β-galactosidase activity coexpressed in these cells. Values are expressed as mean ± SD for three independent experiments (a and b, $P < 0.05$; compared with EV with and without RANKL stimulation, respectively).

These results further suggest that p62^{P392L} enhanced c-Fos gene expression during OCL differentiation. c-Fos plays an important role in expression of NFATc1, a critical transcription factor essential for OCL differentiation and bone resorption. Therefore, we next examined the functional impact of p62^{P392L} in NFATc1 expression during OCL differentiation. Real-time PCR analysis of total RNA isolated from pre-OCL cells showed that p62^{P392L} significantly increased (4.0-fold) NFATc1 mRNA expression without RANKL stimulation compared with p62^{WT} or p62^{A381V} transduced cells. In contrast, no significant changes in NFATc1 expression were observed in p62^{WT} and p62^{A381V} transduced cells compared with EV transduced cells (Fig. 3E).

To further confirm that p62 mutant modulates NF-κB, AP-1, and NFAT transcriptional activity, pNF-κB- Luc, AP-1-Luc, and NFAT-Luc reporter gene plasmids were transfected with p62^{WT}, p62^{A381V}, or p62^{P392L} into RAW 264.7 cells and stimulated with RANKL (100 ng/ml) for 48 h. Total cell lysates obtained from these pre-OCL cells were analyzed for luciferase activity as described in *Materials and Methods*. Mutant p62^{P392L} significantly increased NF-κB, AP-1, and NFAT reporter gene activity with and without RANKL stimulation compared with p62^{WT} and p62^{A381V} transfected cells. However, there was no significant change in the reporter gene activity with p62^{WT} and p62^{A381V} mutant compared with control EV transfected cells (Fig. 4, A–C). These results suggest that the p62 UBA mutant (p62^{P392L}) stimulates downstream effectors of the RANK signaling pathway during OCL differentiation.

p62^{P392L}/CYLD shRNA increase OCL differentiation/bone resorption activity

To determine the potential of p62^{P392L}/CYLD shRNA to stimulate OCL formation, mouse bone marrow-derived nonadherent cells were transduced with CYLD shRNA coexpressed with p62^{WT}, p62^{P392L}, or p62^{A381V} and cultured for OCL differentiation in the presence of RANKL (100 ng/ml) and M-CSF (10 ng/ml) for 5 d. Cells transduced with nonspecific shRNA served as control. The number of TRAP positive multinucleated OCL formed in these cultures was scored. p62^{P392L} significantly increased OCL formation compared with control EV transduced cells. In contrast, no significant change in the rate of OCL formation was observed in p62^{WT} and p62^{A381V} transduced cells (Fig. 5A, *upper panel*). Furthermore, shRNA suppression of CYLD expression significantly increased OCL formation with p62^{WT} and p62^{A381V} similar to p62^{P392L} compared with EV transduced cells (Fig. 5A, *lower panel*). shRNA knockdown of CYLD significantly increased OCL formation compared with nonspecific control shRNA transduced cells, which confirms that CYLD negatively regulates osteoclastogenesis (23). In addition, bone resorption activity of OCL was significantly increased with mutant p62^{P392L} compared with p62^{WT} and p62^{A381V} transduced cells (Fig. 5B, *upper panel*). Also, shRNA suppression of CYLD expression significantly increased bone resorption activity in p62^{WT} and p62^{A381V} similar to mutant p62 ^{P392L} compared with EV transduced cells (Fig. 5B, *lower panel*). Collectively, these results suggest that the p62 UBA mutation (p62^{P392L}) associated with PDB abolished interaction with CYLD, which promotes RANK signaling and increased OCL formation/bone resorption activity (Fig. 6).

Discussion

p62 has been shown to interact with the atypical protein kinase C and TRAF6 to modulate RANK signaling and

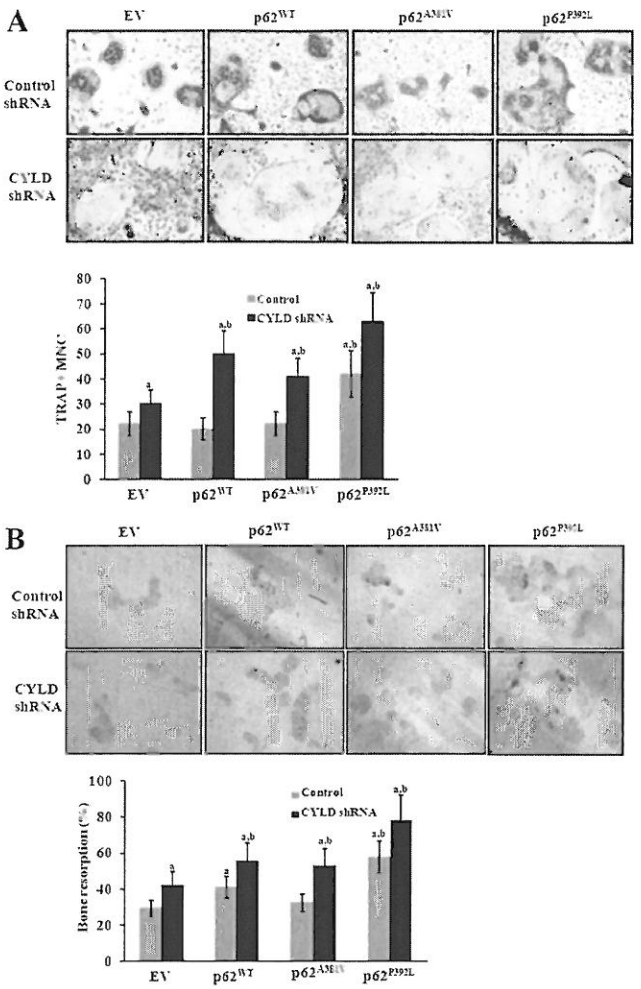

FIG. 5. $p62^{P392L}$/CYLD shRNA stimulation of OCL differentiation in mouse bone marrow cultures. A, Mouse bone marrow-derived nonadherent cells were transduced with CYLD shRNA and coexpressed with $p62^{WT}$, $p62^{A381V}$, or $p62^{P392L}$. Cells were stimulated with RANKL (100 ng/ml) and M-CSF (10 ng/ml) for 5 d, and the TRAP (+) multinucleated OCL formed in these cultures were scored. B, Bone resorption activity of OCL on dentine. The percentage of resorbed area on dentine was quantified as described in *Materials and Methods*. The results represent quadruplicate cultures of three independent experiments (a and b, $P < 0.05$; compared with EV with and without RANKL stimulation, respectively).

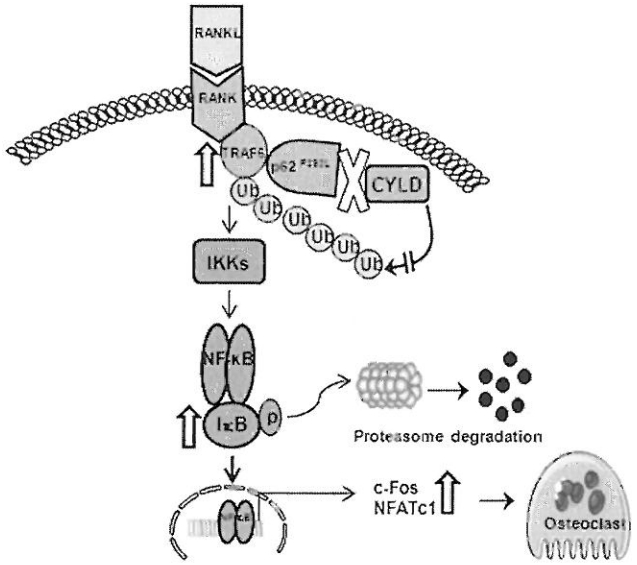

FIG. 6. Schematic illustration of $p62^{P392L}$ mutant regulation of RANK signaling during OCL differentiation. $p62^{P392L}$ mutation widely associated with PDB abolished interaction with CYLD, which promotes RANK signaling through enhanced TRAF6 ubiquitination, c-Fos, and NFATc1 gene expression (as indicated by *open arrows*), and thereby increases the OCL formation/bone resorption activity.

osteoclastogenesis (13). Ubiquitination of RANK adaptor protein, TRAF6, plays an important role in activation of NF-κB during OCL differentiation (31). The UBA domain truncated p62 has been shown to increase RANKL induced NFAT expression and ERK phosphorylation during osteoclastogenesis of RAW 264.7 cells (19). Deubiquitinating enzyme, CYLD, has been shown to inhibit NF-κB activation through deubiquitinatin of TRAF2 and TRAF6 (24). Furthermore, CYLD interacts with the p62 UBA domain to inhibit TRAF6 ubiquitination and negatively regulates RANK signaling and osteoclastogenesis (23). In the present study, we identify that in contrast to $p62^{WT}$ and non-UBA mutant ($p62^{A381V}$), UBA mutant ($p62^{P392L}$) abolished CYLD interaction, and this indicates that RANK signaling essential for osteoclastogenesis is modulated in PDB. We consistently observed accumulation of polyubiquitinated TRAF6 in $p62^{P392L}$ transduced pre-OCL cells.

Ubiquitination of signaling molecules by E3 ubiquitin ligases has been shown to modulate NF-κB signaling (32). Because CYLD negatively regulates different signaling pathways by deubiquitination of K63-linked polyubiquitin chains from several substrates, it is possible that lack of $p62^{P392L}$ interaction with CYLD may affect the ubiquitination status of other RANK signaling molecules that may also play a role in enhanced osteoclastogenesis. Elevated p-IκB expression in $p62^{P392L}$ mutant transduced pre-OCL cells suggests $p62^{P392L}$ activation of NF-κB in these cells. However, a modest increase observed in TRAF6 ubiquitination and p-IκB expression in $p62^{WT}$ and $p62^{A381V}$ compared with control EV transduced cells is more likely due to overexpression of p62 in these cells. Therefore, the mutant $p62^{P392L}$ in contrast to $p62^{WT}$ and $p62^{A381V}$ stimulates NF-κB activity in pre-OCL cells, and this suggests that lack of $p62^{P392L}$ interaction with CYLD may have a specific functional role in enhanced osteoclastogenesis in PDB. Previously, p62 has been shown to be involved in NF-κB activation (13). Further, $p62^{P392L}$ mutation has been shown to elevate NF-κB activation as well as p38 MAPK and ERK1/2 signaling in OCL precursors stimulated with RANKL (17). However, Rea *et al.* (11) have shown that p62 wild type reduces and $p62^{P392L}$ mutant

increases NF-κB activation in HEK293 and Cos-1 cells. Similarly, both p62 non-UBA (A381V) and UBA mutant (P392L) have been shown to increase NF-κB reporter gene activity in HEK293 cells (12). The variability in p62 regulation of NF-κB activity could be due to cell specificity and response to RANKL stimulation. p62 (440 amino acids) contains various domains that mediate protein-protein interactions (33). Several proteins that interact with p62 UBA domain, which includes calmodulin kinase II, nuclear receptor co-receptor I, heat shock protein 70, FK506 binding protein 14, homeobox protein Meis2, and Unc51 like kinase II, have been identified (21). In addition, p62 protein has been shown to interact with several signaling proteins, such as mitogen-activated protein kinases, a protein kinase C, $p56^{lck}$, receptor-interacting protein, TRAF6, ubiquitin, ubiquitinating, and deubiquitnating enzymes (33). Therefore, it is possible that $p62^{A381V}$ may affect interaction with signaling molecules other than CYLD and modulates NF-κB activation in a cell-specific manner. Furthermore, our results that $p62^{P392L}$ expression/shRNA knockdown of CYLD increased NF-κB, AP-1, and NFAT reporter gene activity indicate that mutant $p62^{P392L}$ modulates NF-κB target gene expression required for enhanced osteoclastogenesis in PDB. This is further confirmed by the results that CYLD knockdown significantly increased c-Fos expression in $p62^{WT}$, $p62^{A381V}$, and $p62^{P392L}$ mutant transduced cells without RANKL stimulation. Consistently, up-regulation of c-Fos gene expression in pagetic OCL and osteoblast cells has been reported (34). NFATc1 is a critical transcription factor for osteoclastogenesis (14), and our findings that $p62^{P392L}$ up-regulated NFATc1 expression in pre-OCL cells favors increased osteoclastogenesis associated with PDB. Gene expression profiles in OCL revealed no significant change in p62 and CYLD expression in PDB (35). Inhibition of CYLD resulted in NF-κB activation and apoptotic resistance in hepatocellular carcinoma cells (36). Therefore, it is possible that the $p62^{P392L}$ mutant may have a functional role in proliferation/survival of OCL progenitor cells, which have been shown to be increased in PDB (37). Also B-cell lymphoma 2, antiapoptotic gene expression has been shown to be up-regulated in pagetic bone (38). OCL formed in pagetic bone marrow cultures have high levels of IL-6 and TRAP activity (39). We identified that $p62^{P392L}$ expression increased TRAP mRNA expression in pre-OCL cells (data not shown). $p62^{P392L}$ modulation of c-Fos and NFATc1 expression implicated a potential role in enhanced osteoclatogenesis in PDB. Confirming this, we found that $p62^{P392L}$ expression or shRNA knockdown of CYLD resulted in increased OCL formation and bone resorption activity in mouse bone marrow cultures. Therefore, lack of p62 UBA mutant interaction with CYLD results in increased OCL formation. Mutations in valosin-containing protein, which also contains the UBA domain, have been linked to inclusion body myopathy that is associated with PDB and frontotemporal dementia (IBMFD) (40). Valosin-containing protein is a multiubiquitin chain targeting factor for proteosome degradation, and it also plays an important role in regulating the NF-κB signaling cascade (41). Therefore, identification of $p62^{P392L}$ regulated gene expression profiling is important to better understand the pathogenesis of PDB. Transgenic mice harboring $p62^{P394L}$ mutation (equivalent to human $p62^{P392L}$) showed increase osteoclastogenic potential due to increased RANKL expression in marrow stromal cells in the bone microenvironment. The OCL precursors from these mice also demonstrated increased sensitivity to RANKL but not to 1,25 $(OH)_2D_3$ (42). Furthermore, p62 UBA mutant ($p62^{P394L}$) and MVNP coexpression in mice developed OCL with pagetic phenotype and increased IL-6 production (43). In contrast, others have recently shown that mice with $p62^{P394L}$ mutation have focal lesions with increased OCL number, size, and some nuclear inclusions (18). Consistently, the OCL precursors from these mice are hypersensitive to RANKL. These studies have also suggested that $p62^{P394L}$ mutation up-regulates autophagy, a cellular process for lysosomal degradation of damaged/dysfunctional organelles and protein aggregates (44). However, p62 null mice have a normal skeletal phenotype with no alterations were found in the trabecular size and number of OCL, suggesting that basal osteoclastogenesis is not affected by the loss of p62 (13). Therefore, p62 mutant protein-protein interactions play an important role in enhanced OCL development in PDB.

PDB patients with p62 mutations displayed polyostotic involvement, indicating severity of the disease (45, 46). However, the familial history of PDB is about 15–40% patients with a first degree relative and of which only 20–30% have a p62 mutation. Thus, p62 mutations occur in 5–10% of patients with PDB. Therefore, p62 mutant stimulation of OCL formation/bone resorption is associated with a very limited patient population. Recently, genome-wide association studies in individuals without p62 mutations have further identified genetic variants *CSF1*, *OPTN*, and *TNFRSF11A* as risk factors that predispose to PDB; however,, their functional role in pathogenesis of PDB is yet to be elucidated (47). Presence of nuclear inclusions in Pagetic OCL suggested a viral etiology for PDB; however, no infectious virus is isolated. Despite the controversy about the identity of paramyxoviral nuclear inclusions and MVNP expression in pagetic OCL, it has been shown that targeted expression of MVNP to OCL lineage develops pagetic-like bone lesions in mice (48). Therefore, both genetic and environmental factors, such

as paramyxoviruses, play an important role in pathogenesis of PDB. In conclusions, p62 UBA mutation ($p62^{P392L}$) abolished interaction with CYLD and contributed to enhanced OCL development and excess bone resorption associated with PDB.

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